Reprinted for private circulation from

THE JOURNAL OF INFECTIOUS DISEASES • VOL. 132, NO. 6 • DECEMBER 1975

12

Influenza Alone and in Sequence with Pneumonia Due to *Streptococcus pneumoniae* in the Squirrel Monkey

R. F. Berendt, G. G. Long,[redacted] J. S. Walker

From the U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland

Squirrel monkeys (*Saimiri sciureus*) inoculated intratracheally with $10^{4.2}$–$10^{8.2}$ egg median infectious doses (EID_{50}) of type A influenza virus (H3N2) responded with clinical illness including such signs as fever, sneezing or coughing, coryza, and increased respiratory rates. Necropsy studies performed six days after inoculation revealed bronchopneumonia in addition to a mild tracheitis. Squirrel monkeys given 10^5–6×10^8 colony-forming units (cfu) of *Streptococcus pneumoniae* intratracheally died four to six days later after developing severe illness characterized by fever, bacteremia, lethargy, anorexia, coughing, labored breathing, and bronchopneumonia. Monkeys given 770 cfu of *S. pneumoniae* responded with less severe symptoms and survived. Four squirrel monkeys inoculated with $10^{8.2}$ EID_{50} of virus and then 102 hr later with 770 cfu of *S. pneumoniae* developed severe disease; three of the four animals died within 40 hr. At necropsy these monkeys had more extensive and severe bronchopneumonia than was seen in monkeys infected with either organism alone.

We have previously emphasized the need for a primate model for investigating the sequence of influenza virus infection followed by bacterial pneumonia, and we have reported our inability to establish clinically detectable disease in rhesus monkeys with the virus alone or in sequence with *Streptococcus pneumoniae* [1]. Since that report, we have investigated the responses of several other primate species to influenza virus and *S. pneumoniae*. In this report we present data obtained from an investigation of the sequence of infection in the squirrel monkey (*Saimiri sciureus*) and discuss our results in relation to the human disease syndrome of sequential respiratory infections.

Received for publication March 28, 1975, and in revised form July 24, 1975.

In conducting the research described in this report, we adhered to the "Guide for Laboratory Animal Facilities and Care," as promulgated by the Committee on the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Academy of Sciences–National Research Council. Our facilities are fully accredited by the American Association of Accreditation of Laboratory Animal Care.

We gratefully acknowledge the technical assistance of John M. Kiffmeyer, John E. Young, and Robert D. Magruder. We also acknowledge the valuable advice of Drs. James L. Stookey and James B. Moe.

Please address requests for reprints to Dr. Richard F. Berendt, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, Maryland 21701.

* Present address: Department of Veterinarian Pathology, Washington State University, Pullman, Washington 99163.

Materials and Methods

Organisms. The Aichi/2/68 strain of type A influenza virus (H3N2) was propagated, harvested, and stored as previously described [2]. The methods used for enhancement of virulence and propagation of type I *S. pneumoniae* cultures have also been described previously [1]. The strain employed was that designated no. 6301 by the American Type Culture Collection.

Intratracheal inoculation. Monkeys were anesthetized with 10–15 mg of ketamine hydrochloride (Ketaset,® Bristol Laboratories, Syracuse, N.Y.). A lubricated sterile no. 5 French nasogastric tube (15 cm) containing a wire in the lumen for added rigidity was passed through the mouth into the trachea for a distance of 2–4 cm. To facilitate passage, a pediatric laryngoscope was employed. The wire was withdrawn and the inoculum (0.5 ml at ambient temperature) was slowly administered. The cough reflex of the monkey was not repressed during the procedure.

Animals. Juvenile squirrel monkeys (imported from Bolivia through Primate Imports, Port Washington, N.Y.), weighing 0.3–0.45 kg, were employed. Prior to use, animals were tested by established techniques for serum antibody to the test organisms [1]. Only monkeys with no detectable antibody to either organism (see below) were employed.

Bacterial and viral isolation. Established methods of isolating virus and bacteria from tissues were employed [1, 2].

Serological techniques. The HAI test for antibody to influenza virus and the passive HA test for antibody to type I pneumococcal polysaccharide were performed as previously described [1, 2].

Results

Preliminary experiments. Each of two monkeys was challenged with various doses of either the virus or *S. pneumoniae*. Challenges with $10^{4.2}$–$10^{8.2}$ egg median infectious doses (EID_{50}) of influenza virus produced fever, coryza, rapid respiration, dyspnea, and sneezing or coughing. The day of onset and duration of these signs varied in a manner unrelated to dosage. All monkeys developed serum titers of HAI antibody of $\geq$1:160 by day 14 after inoculation.

Monkeys that received 10^3–6×10^8 cfu of type I *S. pneumoniae* responded with bacteremia, anorexia, lethargy, coughing, dyspnea, and (in the case of monkeys receiving > 1,000 cfu) prostration and death in six to seven days. Fevers varied greatly in duration and time of onset. Doses of 10 cfu did not produce detectable reaction.

Sequential respiratory infection. Information derived from the preliminary studies served as the basis for designing experiments on sequential infection. Each of four squirrel monkeys was given $10^{8.2}$ EID_{50} of influenza virus intratracheally; these four monkeys served as viral controls. Four additional monkeys (designated sequentially infected) were given the same dose of virus and 102 hr later received an intratracheal inoculation of 770 cfu of *S. pneumoniae*. Four other monkeys (pneumococcal controls) were given 770 cfu of *S. pneumoniae* at the same time as the sequentially infected monkeys but received no virus. Four more monkeys (sham controls) were inoculated with sterile heart infusion broth at the time of the other inoculations and 102 hr later and were observed for the duration of the experiment. There were no reactions in the sham controls.

The 102-hr period between viral and bacterial inoculations was based upon the time required for the majority of monkeys receiving influenza virus to develop clinical signs of infection. The viral control and sequentially infected monkeys showed similar clinical signs for the first 102 hr. However, one monkey of the viral control group was severely ill by the morning of the third day, was prostrate by that evening, and died that night.

The principal clinical observations are given in table 1. Respiratory rate was a sensitive indicator of infection. The respiratory rate of viral controls increased sharply after initial challenge, then decreased slowly, and reached normal limits on the fifth day. The sequentially infected monkeys also showed a sharp initial increase in respiratory rate. Another increase was observed after administration of *S. pneumoniae* and persisted until the end of the experiment. In contrast, pneumococcal controls had no increase in respiratory rate. Bacteremia developed the morning after exposure to *S. pneumoniae* in all pneumococcal controls and sequentially infected monkeys.

Anorexia was measured by counting the number of biscuits consumed daily. Anorexia in viral controls occurred only in the monkey that died. None of the pneumococcal controls was anoretic. However, anorexia began in one sequentially infected monkey on day 3 and was seen in all of these monkeys by day 5.

Lethargy was prominent in the sequentially infected group. Three of these four monkeys were unable to climb onto their perches by the fifth day (16 hr after inoculation with *S. pneumoniae*). Occasional coughing was seen in three of the four viral control monkeys (including the one that died) starting on day 3 and continuing through the end of the study. This cough was most pronounced during exertion. In contrast, paroxysmal coughi g was observed in the sequentially infected group within 12 hr after bacterial challenge.

Other than transient neutrophilia that lasted for 24 hr after inoculation in all infected monkeys, leukocyte responses were equivocal.

The experiment was terminated on the morning of the sixth day (42 hr after pneumococcal ex-

Table 1. Response of squirrel monkeys to sequential infection with influenza virus and *Streptococcus pneumoniae*.

Parameter, group*	Day 0	1	2	3	4†	5	6
Bacteremia (% responding)							
VC	0	0	0	0	0	0	0
BC	0	0	0	0	0	100	100
S	0	0	0	0	0	100	100
Anorexia (% responding)							
VC	0	0	0	25	0	0	0
BC	0	0	0	0	0	0	0
S	0	0	0	25	25	75	100
Cough (% responding)							
VC	0	0	0	25	67	67	67
BC	0	0	0	0	0	0	25
S	0	0	0	50	75	100	100
Lethargy (% responding)							
VC	0	0	25	50	100	100	33
BC	0	0	0	0	0	0	0
S	0	0	0	25	75	100	100
Respiration rate (breaths/min)							
VC	111	192	165	164	134	121	121
BC	111	97	103	105	98	112	101
S	111	157	156	159	172	195	231
Cumulative mortality rate (%)							
VC	0	0	0	25	25	25	25
BC	0	0	0	0	0	0	0
S	0	0	0	0	0	0	75

* VC = viral control; BC = bacterial control; S = sequentially infected.
† *S. pneumoniae* was inoculated during the afternoon of day 4.

posure). By this time three sequentially infected monkeys had died (within the previous 6 hr) and the fourth was very ill. The survivors (one monkey infected sequentially, two pneumococcal controls, and three viral controls) were killed with pentobarbital and necropsied. Pneumococci were recovered in small numbers (<100/ml or /g) from the blood and lungs of bacterial controls and in large numbers (>100,000/ml or /g) from sequentially infected monkeys at the time of death. The mean numbers of bacteria and viruses isolated from blood and lungs at necropsy are given in table 2. Influenza virus was isolated from the lungs but not the blood of both viral controls and sequentially infected monkeys. Slightly higher quantities of virus were recovered from lung tissue of sequentially infected monkeys than from lungs of controls.

Pathology. (1) Influenza virus control monkeys. Of the four monkeys in this group, three had patchy, plum-red to gray consolidation involving about one-eighth of the total surface area of the lung. The single fatal case in this group had patchy, plum-red foci of consolidation with poorly defined borders throughout the lungs; about one-half of the total lung surface area was involved.

The typical histologic changes in the trachea and main bronchi were loss of cilia, minimal distortion of the mucosa, and minimal polymorphonuclear neutrophilic infiltration of the submucosa. The changes in the lungs of three surviving monkeys consisted of small focal areas of infiltration of the alveolar septa by macrophages and a few polymorphonuclear neutrophils, accompanied by hyperplasia of alveolar and bronchiolar epithelium. In some areas, alveoli adjacent to bronchioles contained an exudate of macrophages, neutrophils, and lymphocytes in a pale eosinophilic homogeneous material. Peribronchiolar and perivascular foci of lymphocytes with small numbers of macrophages were also seen.

In the single fatal case, changes were suggestive

Table 2. Recovery of influenza virus and *Streptococcus pneumoniae* from monkeys at necropsy.

Organism, group*	Mean concentration in†	
	Blood	Lung
Influenza virus		
VC	0	$10^{1.0}$
BC	0	0
S	0	$10^{2.7}$
Streptococcus pneumoniae		
VC	0	0
BC	5.5×10^1	9.0×10^1
S	3.0×10^4	$>1 \times 10^5$

* VC = viral control; BC = bacterial control; S = sequentially infected.

† For influenza virus, mean concentrations are expressed in egg median infectious doses/ml; for *S. pneumoniae* concentrations are expressed in cfu/ml.

of a bacterial etiology, but culture of the lungs was not attempted and no bacteria were seen microscopically.

(2) Pneumococcal control monkeys. Two monkeys in this group were killed and necropsied. No significant gross or histological changes were seen.

(3) Sequentially infected monkeys. The lungs of all sequentially infected monkeys contained lobes that were completely or largely consolidated and were red-gray to gray-brown in color. The predominant changes occurred in lobes of the left lung of two monkeys and the right lung of the other two. Severely affected areas were covered with a fibrinous pleural exudate. The remainder of the lungs of all monkeys contained minimal to prominent amounts of patchy red consolidation.

Microscopic examination disclosed widespread distention of alveoli, with pale, homogeneous, eosinophilic material containing neutrophils and fibrin. Bacterial cocci were noted within the exudate. Some alveolar septa were necrotic. Bronchioles contained plugs of neutrophils and necrotic debris, and some bronchiolar walls were not distinguishable. Alveolar and bronchiolar epithelial hyperplasia was noted in some areas. In severely affected lobes, lymphatics were greatly distended with fibrino-purulent necrotic debris. Perivascular edema was present in septal tissue. The pleura overlying severely affected areas was thickened and infiltrated with polymorphonuclear leukocytes and fibrinous exudate.

Two monkeys had histological evidence of bacteremia. In one monkey the reaction was confined to the liver, with small foci of necrotic hepatocytes and minimal leukocytic infiltration associated with gram-positive cocci; minimal infiltration of leukocytes in liver, gall bladder, adrenals, and bronchial lymph node was also observed. Bacteria were also observed in the glomeruli of the kidney.

S. pneumoniae-induced pathology. The results obtained in both preliminary and sequential-infection experiments suggested that *S. pneumoniae* did not cause pneumonia when administered alone. To determine the histological changes associated with these infections, four monkeys were inoculated intratracheally, two with 10^6 cfu and two with 10^3 cfu. The animals receiving the larger dose died on day 4 and were necropsied; the monkeys receiving the smaller dose were sick on day 5 and were sacrificed by iv inoculation of pentobarbital and necropsied. Histological changes were more extensive in monkeys receiving the larger dose, but in both groups the most prominent findings were related to the consequences of bacteremia rather than to multiplication of *S. pneumoniae* in the lung. Only minimal pneumonitis was seen in monkeys that received 10^3 cfu, a dose equivalent to that employed in the experiment involving sequential infection.

Discussion

Squirrel monkeys develop clinical illness after intratracheal challenge with small doses of influenza virus or *S. pneumoniae* but suffer a severe disease when the two organisms are given sequentially. This reaction confirms the many reports of experimentally produced synergism between virus and various bacteria; such synergism has been reported in rhesus monkeys by Wilson et al. [3]; in mice by Harford et al. [4], Harford and Hara [5], Gerone et al. [6], and Sellers et al. [7]; and in guinea pigs by Janssen et al. [8]. The naturally occurring sequence in human patients has been described in many reports in standard texts.

The clinical and pathological observations in monkeys infected with influenza virus are not inconsistent with those reported in human patients [9, 10], although the disease in squirrel monkeys seems to involve the lungs to a greater extent than has been thought to be the case in uncomplicated human influenza. However, recent observations

in humans suggest that involvement of the small airways is more common than was previously thought [11].

Since administration of *S. pneumoniae* alone into the trachea was followed by extensive bacteremia rather than by severe pneumonia, the squirrel monkey probably cannot be used as a model for human pneumococcal pneumonia.

There is no doubt that prior influenza infection increased the replication of *S. pneumoniae* in the lungs and that severe pneumonia was produced. However, intranasal inoculation of benign substances such as saline solution has been shown to exacerbate respiratory influenza infections in mice [12]; therefore, it will be necessary to determine the role of virus in the clinical and pathological reactions that occur after bacterial inoculation.

Since the squirrel monkey appears to develop a reproducible illness after exposure to influenza virus, this subhuman primate may serve a useful role in the study of sequential infections with other bacteria.

References

1. Berendt, R. F., McDonough, W. E., Walker, J. S. Persistence of *Diplococcus pneumoniae* after influenza virus infection in *Macaca mulatta*. Infec. Immun. 10:369–374, 1974.
2. Berendt, R. F. Simian model for the evaluation of immunity to influenza. Infec. Immun. 9:101–105, 1974.
3. Wilson, H. E., Saslaw, S., Doan, C. A., Woolpert, O. C., Schwab, J. L. Reactions of monkeys to experimental mixed influenza and streptococcus infections. An analysis of the relative roles of humoral and cellular immunity, with the description of an intercurrent nephritic syndrome. J. Exp. Med. 85:199–215, 1947.
4. Harford, C. G., Leidler, V., Hara, M. Effect of the lesion due to influenza virus on the resistance of mice to inhaled pneumococci. J. Exp. Med. 89: 53–68, 1949.
5. Harford, C. G., Hara, M. Pulmonary edema in influenzal pneumonia of the mouse and relation of fluid in the lung to the inception of pneumococcal pneumonia. J. Exp. Med. 91:245–260, 1950.
6. Gerone, P. J., Ward, T. G., Chappell, W. A. Combined infections in mice with influenza virus and *Diplococcus pneumoniae*. Am. J. Hyg. 66:331–341, 1957.
7. Sellers, T. F., Schulman, J., Bouvier, C., McCune, R., Kilbourne, E. D. The influence of influenza virus infection on exogenous staphylococcal and endogenous murine bacterial infection of the bronchopulmonary tissues of mice. J. Exp. Med. 114:237–256, 1961.
8. Janssen, R. J., Chappell, W. A., Gerone, P. J. Synergistic activity between PR8 influenza virus and *Staphylococcus aureus* in the guinea pig. Am. J. Hyg. 78:275–284, 1963.
9. Knight, V., Kasel, J. A. Influenza viruses. *In* V. Knight [ed.]. Viral and mycoplasmal infections of the respiratory tract. Lea and Febiger, Philadelphia, 1973, p. 87–123.
10. Hers, J. F. Ph., Masurel, N., Mulder, J. Bacteriology and histopathology of the respiratory tract and lungs in fatal Asian influenza. Lancet 2:1141–1143, 1958.
11. Douglas, R. G., Jr., Alford, R. H., Cate, T. R., Couch, R. B. The leukocyte response during viral respiratory illness in man. Ann. Intern. Med. 64:521–530, 1966.
12. Taylor, R. M. Experimental infection with influenza A virus in mice. The increase in intrapulmonary virus after inoculation and the influence of various factors thereon. J. Exp. Med. 73:43–55, 1941.